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(54) Title: SYNTHETIC PEPTIDES OF HUMAN PAPILLOMAVIRUS

Asn	Leu	Ala	Ser	Ser	Asn	Tyr	Phe	Pro	Thr	(1)
1				5					10	
Pro	Ser	Gly	Ser	Met						
				15						

Pro	Ser	Gly	Ser	Met	Val	Thr	Ser	Asp	Ala	(2)
1				5					10	
Gln	Ile	Phe	Asn	Lys						
				15						

Gln	Ile	Phe	Asn	Lys	Pro	Tyr	Trp	Leu	Gln	(3)
1				5					10	
Arg	Ala	Gln	Gly	His						
				15						

(57) Abstract

Peptides for conjoint use in therapy of patients having an infection caused by HPV 16 or a like human papillomavirus, or in which such an HPV has been implicated, especially cervical dysplasias, said peptides being from 15 to 24 amino acids long and consisting of or including the following sequences of amino acids of human papillomavirus (HPV) 16 L1 protein: (1) SEQ ID. NO: 1 = aa residues 311-325, or a conservatively modified variant thereof; (2) SEQ ID. NO: 2 = aa residues 321-335, or a conservatively modified variant thereof; and (3) SEQ. ID. NO: 3 = aa residues 331-345, or a conservatively modified variant thereof. Compositions of these three peptides *per se* or for use in such therapy are included in the invention.

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SYNTHETIC PEPTIDES OF HUMAN PAPILLOMAVIRUS

Background of the Invention

(1) Field of the Invention

The present invention provides synthetic peptides identified in human papillomavirus (HPV) L1 proteins which are useful in the prevention and treatment of genital warts, cervical dysplasias and cervical cancer.

(2) Description of the related art

Human papillamoviruses are a group of viruses which share a common genetic organisation with animal papillomaviruses and which induce cutaneous and mucosal squamous epithelial lesions. Over 65 different HPV types have been identified.

Different types of HPV cause different diseases and appear at different sites in the body. Several types, in particular type 6, 11, 16, 18, 31, 33, 35 and 52, infect the genital region. The HPV types 6 and 11 mainly cause genital warts, known as condyloma acuminata. Other HPV types 16, 18, 31, 33, 35, 39, 51 and 55 cause mainly flat warts and dysplastic lesions of the cervical epithelium which are histologically defined as cervical intraepithelial neoplasia (CIN). These CIN-lesions can develop further to non-invasive carcinoma (carcinoma in-situ) or invasive squamous cell carcinoma. More than 90% of all cervical cancers have genetic evidence of some type of HPV integration into the host genome of which HPV 16 alone is the most commonly found (60% of all cervical tumours) and is the type of HPV of most interest in the current application. The relationship of HPV infection to the development of CIN and cervical carcinoma is unclear, however it has been postulated that HPV acts as an initiator in cervical carcinogenesis and that malignant transformation depends on interaction with other factors (Zur Hausen, H. et al, Lancet, (1982) ii:1370).

The nature of HPV 16 in particular, and papillomaviruses in general has been well studied. HPV 16 contains a 7904 bp double-stranded DNA genome (Siedorf. K. et al, Virology,

(1985) 145: 181-185). The capsid is 50 nm and contains 72 capsomers (Klug. A., J Mol. Biol., (1965), 11:403-423). A number of HPV 16 subtypes have been reported which are isolates showing greater than 50% homology (Coggin, Cancer Research, (1979), 39:545-546), but give differences in restriction fragment length polymorphism patterns with different restriction endonucleases on Southern Blotting.

All HPV genomes have at least 8 regions which code for a number of proteins as transcripts from open reading frames (ORF). These regions are functionally divided into early regions, postulated to encode the proteins needed for replication and transformation (E1 to E7) and late regions, which encode the two major viral capsid proteins (L1 and L2). The late regions show regions of homology in all HPV types.

Little is known about the immune response to infections by HPV 16 and papillomaviruses in general. The detection of HPV in, for example, cervical samples can be carried out using sensitive DNA typing techniques such as the Polymerase Chain Reaction and Southern Blotting, but the virus cannot be isolated and grown in the laboratory to adequate amounts of antigenic material suitable for immunological testing (Tichman et al., J. Invest. Derm., 1984, 83: 25-65).

Seroreactive epitopes of HPV have been identified and antibodies raised against them. These antibodies find use in the diagnosis of HPV infections, and for example are described in WO91/18294 (Dillner and Dillner) wherein synthetic peptides representing the L1 and L2 proteins of HPV 16 were synthesised, and tested with human sera. The synthetic peptides against which these antibodies reacted were also proposed as being useful in diagnosis of HPV infections. However, no therapeutic use of these antibodies or peptides was suggested.

In the absence of a suitable in vitro bioassay system to screen for biologically important B and T-cell epitopes, it is not possible to define whether these and other reported antibodies with HPV binding activity in Enzyme Linked Immunoassays (ELISAs) have HPV neutralising ability.

Additionally, it is by no means predictable that a seroreactive epitope against which an antibody with potential use in diagnosis may be raised (a B-cell epitope) is itself an epitope which is capable of eliciting a protective immune response. Upon infection by a foreign antigen such as HPV, the immune system would be expected to mount specific T-cell and B-cell responses to a large number of antigenic determinants recognised on the different HPV antigens presented to the host via antigen presenting cells (APCs).

European Patent Applications 257 754 (Schoolnik), 412 762 (Oliff) 451 550 (Muller) and Muller et al., (J. Gen. Virol., 1990, 71:2709-2717) describe epitopes of HPV, including HPV type 16 which they say are useful in therapy. Of all these epitopes described in the art, the majority are concerned with proteins of the early region. Muller also discloses an 88 amino acid sequence from HPV 16 L1 which is useful in vaccines. However in all cases, the evidence is based upon serological tests, which suggests that they are looking at B-cell epitopes and there is no evidence provided to show that that these peptides are sufficiently immunogenic to produce a B-cell response, and whether the resulting B-cell antibodies are HPV neutralising antibodies and are thus protective against HPV infection.

T-cell epitopes are also important in the immune response of individuals to an invading antigen. T-cells, together with macrophages and polymorphonuclear leucocytes, are the body's first and main line of immune defence.

P Shepherd (the present inventor) and co-workers have tested the immune response to certain peptides derived from HPV 16.L1 in a T-cell proliferation assay designed to measure the ability of these peptides to specifically stimulate T-cells taken from patients with cervical dysplasias to proliferate. Various disclosures at meetings took place in 1992 and 1993, by which it was shown that the patients' cells were stimulated best by either one or both of two pools of peptides, one pool containing two

peptides from amino acid residues 305-329, the other containing three peptides from aa residues 321-345.

It is notoriously difficult to define accurately peptides representing epitopes and even more difficult to define a set of relatively short peptides which together will provide T-cell stimulatory responses in a high proportion of patients having the relevant clinical symptoms, in this case cervical dysplasias. Long peptides are to be avoided, both from the point of view of cost of synthesis, and from the proper presentation of those peptides by antigen-presenting cells of the patient. The present invention provides a solution to this problem by defining a set of peptides recognised by most patients within a test group.

Summary of the invention

The invention provides the three peptides for conjoint use in therapy of patients having an infection caused by HPV 16 or a like human papillomavirus, or in which such a HPV has been implicated, especially a human papillomavirus, particularly cervical dysplasias, said peptides being from 15 to 24 amino acids long and consisting of or including the following sequences:

(1) SEQ ID. NO:1 = aa residues 311-325:

Asn	Leu	Ala	Ser	Ser	Asn	Tyr	Phe	Pro	Thr
1				5					10
Pro	Ser	Gly	Ser	Met					
				15					

or a conservatively modified variant thereof;

(2) SEQ ID. NO: 2 = aa residues 321-335:

Pro	Ser	Gly	Ser	Met	Val	Thr	Ser	Asp	Ala
1				5					10
Gln	Ile	Phe	Asn	Lys					
				15					

or a conservatively modified variant thereof; and

(3) SEQ ID. NO: 3 = aa residues 331-345:

Gln	Ile	Phe	Asn	Lys	Pro	Tyr	Trp	Leu	Gln
1				5					10
Arg	Ala	Gln	Gly	His					
				15					

or a conservatively modified variant thereof.

The invention also includes a therapeutic composition per se or for use in therapy as defined above, comprising the three peptides as defined above.

Nothing in the above definition excludes the use in therapy or co-presence in the composition of other peptides or other compatible active principles or other ingredients. Further, the peptides can in each case be free or bound (including bound by one or more covalent chemical bonds) to any carrier or assistant molecule which may facilitate, ameliorate or at least be compatible with the above-defined therapeutic purpose.

The invention includes the possibility of replacing two or all three of the above peptides by a single peptide of the same length as or longer than required to span the sequence represented by the combination of two or three peptides. However, the benefits of using the shorter peptides make this course unattractive. In that event, however, the peptide(s) should not cover HPV 16 L1 sequence outside the 311-345 range, i.e. beyond the N-terminus at 311 or the C-terminus at 345.

Description of the Preferred Embodiments

The peptides for the use in the invention are those peptides which are capable of eliciting a T-cell response. The precise make up of the sequence necessary for an optimum T-cell response may vary from individual to individual. By combining peptides of the invention, which are preferably 15 to 22, most preferably 15 to 20 and still more preferably 15 to 18 aa long, due allowance can be made for the diversity of T-cell epitope recognition within the human population. Any additional amino acids, beyond the mandatory length of 15 set forth above, are preferably the contiguous amino acids of HPV 16.L1, especially 305-311 for peptide (1).

It is believed that the peptides for use in the invention stimulate the production of T-helper cells which in turn provide helper functions and cell to cell signal transduction to specific B-cells to produce antigen/peptide and cytokine release, HPV-specific antibody responses and/or to help cytotoxic T-cells

to kill or deactivate the virus or virally infected cells. Thus T-helper cells play an important pivotal role in co-ordinating the body's two main immune defence mechanisms. Hence, the inclusion of T-helper cell epitopes in a system used for the presentation of antigens such as B-cell epitopes or cytotoxic T-cell epitopes may, by the added stimulation of the production of T-helper cells, lead to an improved response to the B-cell epitope or cytotoxic T-cell epitope which is being presented. Thus, according to a preferred feature of the invention, a peptide defined hereinbefore is linked to a B-cell or cytotoxic T-cell epitope.

The B-cell epitope linked to the peptides for use in the invention is of preferably from HPV especially types 16,11 and 6 and more preferably HPV 16 but could be any other preferred antigen/peptide to which it is desired to generate an immune response. The B-cell epitope may be from either the early regions (E1 to E7) or, more preferably, the late regions (L1 and L2) of HPV. There may be more than one B-cell or cytotoxic T-cell epitope present together with more than one peptide of the invention.

As used herein, the term "peptide" includes neutral (uncharged) and salt forms, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications. It is well understood in the art that amino acid sequences contain acidic and basic groups, and that the particular ionization state exhibited by the peptide is dependent on the pH of the surrounding medium when the peptide is in solution, or that of the medium from which it was obtained if the peptide is in solid form. Also included in the definition are peptides modified by additional substituents attached to the amino acid side chains, such as glycosyl units, lipids, or inorganic ions such as phosphates, as well as modifications relating to chemical conversions of the chains, such as oxidation of sulfhydryl groups. Thus, the term "peptide" includes a molecule of the appropriate amino acid sequence defined above,

subject to those of the above-described modifications which do not destroy its immunogenic properties.

The peptides for use in the invention may be prepared by chemical synthesis using standard techniques in the art of peptide synthesis.

It is well recognised in the art that replacing one amino acid by another with similar properties, e.g. replacing an aspartic acid residue by a glutamic acid residue may not affect the properties of the peptide in which such a substitution has occurred. Therefore, the invention extends to conservatively modified variants of the peptides in which such substitutions have been made, without affecting the properties of the peptides. In particular it extends to replacing 1, 2 or 3 of the amino acids of SEQ ID NOS. 1-3, by other amino acids within the same category of non-polar, uncharged polar, positively charged or negatively charged. Ala, Val, Leu, Ile, Pro, Phe, Trp and Met are non-polar; Gly, Ser, Thr, Cys, Tyr, Asn and Gln are uncharged polar; Lys, Arg and His are positively charged and Asp and Glu are negatively charged.

The peptides listed above may have various other chemical modifications made to them and still be within the scope of the present invention. In particular, the peptides may be prepared with or without an amide group at the C-terminus. When prepared with an amide group at its C-terminus, the peptides have a carboxy-terminal amide group covalently linked to the carbonyl moiety of the preceding amino acid residue. The peptides may also have an acetyl group covalently attached to the amino acid at the N-terminus. Other chemical modifications are possible, particularly cyclic and dimeric configurations.

The peptides for use in the invention may be used alone, or being of low molecular weight, may be attached to an immunogenic carrier material in order to further stimulate their antigenicity. According to another feature of the invention, conjugate peptides as described hereinbefore, which may be linked to a B-cell or cytotoxic T-cell epitope as described above,

together with an immunogenic carrier material, are used in the invention.

The term "immunogenic carrier material" as used in this specification includes those materials which have the property of independently eliciting an immunogenic response in a host animal and which can be covalently coupled to peptides either directly via a formation of peptide or ester bonds between free carboxyl, amino or hydroxyl groups in the polypeptide and corresponding groups on the immunogenic carrier material or alternatively by bonding through a conventional bifunctional linking group. Examples of such carriers include albumins of animal sera, globulins of animal sera, thyroglobulins of animals, haemoglobins of animals, haemocyanins of animals, particularly Keyhole Limpet Haemocyanin (KLH), proteins extracted from ascaris (ascaris extracts, such as those described in Japanese Laid-Open Patent Application No. 16,414/81, J. Immunology, 111, 260-268 (1973), J. Immunology, 122, 302-308 (1979), J. Immunology, 98, 893-900 (1967) and Am. J. Physiol. 199, 575-578 (1960) or purified products thereof); polylysine, polyglutamic acid, lysine-glutamic acid copolymers, copolymers, containing lysine or ornithine, etc. Recently, vaccines have been produced using diphtheria toxoid or tetanus toxoid as immunogenic carrier materials [Lepow. M.L., et al., J. Infectious Diseases, 150, 402-406 (1984); and Coen Beuvery, E., et al., Infection and Immunity, 40, 39-45 (1983)] and these toxoid materials can also be used herein. Other suitable carriers are disclosed in, for example, US Patent 4,575,495, including vaccines, organic polymers etc. The protein derivative of tuberculin (PPD) is particularly preferred for utilisation in the "active" immunisation scheme since (1) it does not induce a T-cell response itself (i.e. it is in effect a "T-cell hapten"), and yet it behaves as a fully processed antigen and is recognised by T-cells as such; (2) it is known to be one of the most powerful hapten "carriers" in the linked recognition mode; and (3) most importantly, it can be used in humans without further testing.

As peptide-carrier binding agents, those conventionally employed in the preparation of antigens can be widely employed.

The covalent coupling of the peptide to the immunogenic carrier material can be carried out in a manner well known in the art. Thus, for example, for direct covalent coupling it is possible to utilise a carbodiimide, most preferably dicyclohexylcarbodiimide or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, as coupling agent. Glutaraldehyde may also be used as a means of the covalent coupling of the peptide to the immunogenic carrier material.

In the above, proportions of the peptide, peptide-carrier binding agent and immunogenic carrier material can be appropriately determined, but it is preferred that the carrier be employed in an amount of about 1 to 6 times, preferably about 1 to 5 times the weight of the peptide and the peptide-carrier binding agent be employed in an amount of about 5 to about 10 molar times the amount of the peptide. By the above reaction, the carrier is bound to the peptide via the peptide-carrier binding agent to obtain a desire antigen composed of a peptide-carrier complex.

After completion of the reaction, the thus obtained conjugate can easily be isolated and purified by means of a dialysis method, a gel filtration method, a fractionation precipitation method, etc.

The peptides for use in the present invention find utility for the prevention or treatment of genital warts, cervical cancer, especially the early signs thereof manifested by dysplasias, or other conditions caused by HPV in man.

The amino acid sequences of the L1 region of all types of HPV are highly conserved. The term "like human papillomavirus" in the definition of the invention includes those which have 50% or greater identity, especially 65% or greater identity of amino acid sequence - with respect to amino acids 311-345 of HPV 16 L1. The peptides for use in the present invention may find utility in the prevention and treatment of many HPV associated diseases,

especially those caused by HPV 6, and 11, but most especially HPV 16. The primary intended use is on patients having cervical dysplasias, as these are frequently associated with HPV 16 infection.

Direct administration of the peptides for use in the invention to the host can confer protective immunity to the host against HPV, or, if the subject is already infected and thus immunologically primed act as a boost to the subjects own specific HPV immune response to promote a more effective defence against the progress of their HPV or HPV-related disease. The peptides of the present invention may be included in pharmaceutical compositions for the treatment or prevention of diseases involving HPV.

In a pharmaceutical composition which comprises a therapeutically effective amount of a peptide, peptide linked to a B-cell or cytotoxic T-cell epitope or a peptide conjugate as described hereinbefore or a pharmaceutically acceptable salt thereof together with a pharmaceutically acceptable diluent or carrier, other therapeutic ingredients may also be included. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic organic and inorganic bases. The compositions include compositions suitable for oral, topical (including vaginal) or parenteral (including subcutaneous, submucosal, intramuscular, intravenous and intra-arterial) administration, although the most suitable route in any given case will depend on the nature and severity of the conditions being treated and on the nature of the active ingredient. They may be conveniently presented in unit dosage form and prepared by any of the methods well-known in the art of pharmacy.

In practical use, peptides for use in this invention can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms, depending on the form of preparation desired for

administration. In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water glycols, oils, alcohols, flavouring agents, preservatives, colouring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, capsules and tablets. Because of their ease of administration, tables and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar coating or enteric coated by standard techniques.

Pharmaceutical compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tables each containing a predetermined amount of the active ingredient, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion.

Suitable topical formulations include transdermal devices, aerosols, creams, ointments, lotions, dusting powder, and the like.

Preferably however, the peptides are to be administered parenterally, more preferably as vaccines. If desired, the vaccines include some form of adjuvant for stimulating the response to the antigenic material. Examples of adjuvant materials include aluminium hydroxide and saponin.

Preparation of vaccines which contain peptide sequences as active ingredients are well understood in the art. Preferably the vaccines are prepared as injectable forms and are administered preferably by injection, for example intravenously, subcutaneously, intramucosally, intraepidermally or intradermally, preferably intramucosally.

The compositions may be formulated in unit dosage form, or a multiple or a submultiple of a unit dose. Additionally the vaccine may be in a form which provides delayed release of the peptides of peptide conjugated with time.

The vaccines are administered in a manner compatible with the dosage formulation and in such amount as will be therapeutically effective and immunogenic. The quantity to be treated, the capacity of the subject's immune system to synthesise antibodies and the degree of protection desired. Precise amounts depend upon various factors. Whilst the dosage of active compound given will depend upon various factors, by way of guidance the dosage will usually be 0.1 mg to 20 mg/kg body weight. Where national patent law permits, the present invention also includes a method of preventing or treating HPV infections in the human body, which comprises administering the peptides for use in the invention to a human patient in a therapeutically effective dose, e.g. in the range 0.1-20 mmole/kg body weight, preferably 1-2 mmole/kg, administered daily or twice daily during the course of treatment. Alternatively, the invention includes the compounds of the invention for use in said therapy and their use in the manufacture of medicaments for that purpose.

In addition to the common dosage forms set out above, the peptides may also be administered by controlled release means and/or delivery devices.

The following Examples illustrate the invention.

Example

Synthesis of Synthetic Peptides of HPV16 L1

Synthetic peptides were synthesised by the standard F-moc method of peptide synthesis. They had the following aa sequences from HPV 16 L1:

(4) 301-315; (5) 305-319; (6) 311-319; (7) 311-325; (8) 314-329; (9) 321-335; (10) 325-335; (11) 331-345; and (12) 335-345.

Synthesis/Preparation of HPV16 L1 Fusion Protein

The HPV16 L1 fusion protein was prepared by the method described by Patel et al., J. Gen. Virol., 1989, 70, 69-77.

Derivation of short term lines from patients with cervical dysplasias

(a) Materials and Methods

Reagents;

Hanks BSS (HBSS) (042-04060H; Life Technologies Ltd, Paisley, Scotland)

Lymphoprep (Nycomed, Oslo, Norway)

Dimethylsulphoxide (10323; BDH, Poole, UK)

Tissue culture medium: RPMI 1640 with L-glutamine (041-01875M: Life Technologies Ltd, Paisley, Scotland), supplemented with:- 1mM sodium pyruvate (16-820-49); Flow Laboratories, Irvine, Scotland) 2mm L-glutamine (043-05030H: Life Technologies, Paisley, Scotland). 10mM HEPES (44285; BDH, Poole, UK) 100µ/ml of penicillin, 25µg/ml of gentamycin (16-762-45; Flow Laboratories, Irvine, Scotland), 50µM 2-mercaptoethanol (M-6250; Sigma, Poole, UK), and 0.25µg/ml "Fungizone" Registered Trademark) (16-723-46; Flow Laboratories).

Heat inactivated pooled human serum (n=20)

(b) Patient lymphocyte preparations

50ml of venous blood was taken from each patient and defibrinated in a 50ml centrifuge tube (Cel-Cult;) containing 15 glass balls (332124G; BDH, Poole, UK), by rotating at room temperature for 5 mins. The blood was decanted into a fresh 50ml centrifuge tube and spun at 1642g for 10 mins at room temperature. The serum was collected and the packed blood cells resuspended in 100ml HBSS. 15ml aliquots of Lymphoprep were put into 4 x 50ml centrifuge tubes and 25ml diluted blood layered carefully onto each. After spinning at 942g for 30 minutes at room temperature, the cells at the interface were collected and washed with an equal volume of HBSS at 350g for 10 mins at room temperature. The cell pellets were resuspended in 10mls HBSS and counted with yields of from $4.5x10^7$ to $6x10^7$ cells per patient. These cells were spun at 350g for 10 mins at room temperature and then resuspended in 4.5ml autologous serum containing 10% dimethylsulphoxide. 1.5ml aliquots of this mixture were transferred to liquid nitrogen storage.

Short term lines

These were set up using one of the three vials of stored patient cells. The cells were rapidly thawed in a 37°C water bath and then diluted to 25ml in culture medium and spun at 350g for 10 mins at room temperature. The pelleted cells were then resuspended in 2ml of culture medium containing 10% autologous serum, counted and the cell numbers adjusted to $2x10^6$/ml. The fusion protein of HPV16 L1 (amino acid residues 199-409) was diluted in culture medium containing 10% autologous serum and 100µl (1µ/ml) was dispensed into 30 wells of a 96-well round-bottomed microtitre plate. 100µl of the cell suspension (2 x 10^5 cells) was added to each well and the plates incubated at 37°C in a humidified 5% CO_2 atmosphere for 14 days. The cell cultures were fed on days 3 and 7 by removing 100µl of supernatant and replacing it with medium containing 5% autologous serum and recombinant IL2 (25 units/ml, Sandoz) and recombinant IL4 (4 units/ml, Glaxo), and on day 11 with medium containing 5% serum only.

Specificity assays on short term lines

Antigen specificity assays were performed on day 14. Twenty-five short term lines were prepared and 20 were tested per patient. Each of the 20 short term lines was tested against HPV16 L1 fusion protein (1µg/ml); ß-galactosidase (G-6008, Sigma, UK) 10µg/ml; each of the nine HPV16 L1 synthetic peptides (4) to (12) referred to above and culture medium. Peptides were used at 10µM in all assays. Cell counts were performed on 5 pooled cell lines and the mean counts used to estimate the numbers of cells per line ($\pm 2x10^5$ cells). Each cell line was set up in duplicate in 96-well round-bottomed microtitre plates against the 11 antigens listed above and one medium control in the presence of autologous antigen-presenting cells (APC). The latter were from the two remaining vials of the patients' cells stored in liquid nitrogen which were thawed, washed and irradiated (4000 Rads) within 2 hours of thawing. APC numbers varied between patients ($\pm 2.5 \times 10^7$ cells) and were used in the assays at $2.5x10^5$ml.

The peptides were dispensed in medium (100µl/well) to which were added APCs ($2.5x10^5$ml) and line cells (2-5x10 ml) also in 100µl of medium. The plates were incubated for three days at 37°C in 5% CO_2 before adding 20µl of [Methyl-^{3}H] thymidine (TRA 120, Amersham; 9.25KBq/well) and incubating for a further 18h. Incorporation of labelled thymidine was measured by counting the harvested wells in a liquid scintillation β-spectrometer (LKB). The data were plotted as disintegrations per minute (dpm) for all the antigens and medium controls for each cell line and a positive response was taken as a stimulation index (SI) of > 2.5 that of the medium control for each line and having a Δ dpm (the difference in dpm over the background) of >500. The positive response was considered significant when ten percent or more of the short term lines, i.e. 2 or more of the 20 lines, responded positively to the antigen.

The proliferative T-cell responses of short term lines from 24 patients to β-gal HPV 16.L1 fusion protein (aa 199-409) and to synthetic peptides (4) to (12) was tested and compared to 8 disease-free control subjects. The results are tabulated in Table 1 below.

TABLE 1

Proliferative T-cell responses of patients' short term lines to β-gal HPV 16.L1 fusion protein (aa 199-409) and to synthetic peptides (4) to (12). 24 patients and 8 control subjects tested.

Stimulating antigen	Number of subjects giving >10% response: Patients	Controls
β-gal HPV 16.L1	24/24	8/8
Control patients tested against all the above peptides.		0/8
(301-315) on its own	0/24	0/8
(305-319) " " "	0/24	0/8
(311-319) " " "	0/24	0/8
(311-325) " " "	1/24	0/8
(315-329) " " "	0/24	0/8
(321-335) " " "	1/24	0/8
(325-335) " " "	0/24	0/8
(331-345) " " "	3/24	1/8
(335-345) " " "	0/24	0/8
(311-325) + (321-335) only	2/24	0/8
(311-325) + (331-345) only	1/24	0/8
(321-335) + (331-345) only	4/24	0/8
(311-325) + (321-335) + (331-345) only	4/24	0/8
TOTAL RESPONSES:	16/24 = 66%	1/8

Summary of major responses

(311-325) alone or in combination with (321-335) and/or (331-345)	8/24
(321-335) alone or in combination with (311-325) and/or (331-345)	11/24
(331-345) alone or in combination with (311-325) and/or (321-335)	12/24

The results shown in the Table show that 16/24 patients proliferated to one or more of peptides 311-325, 321-335 and 331-345. These three peptides overlap at aa 321-325 and 331-335, but, evidently, these must be presented in the context of preceding and following sequences respectively. Only one patient responded to peptide 321-335 alone. Nor was the combination 311-325 with 331-345 adequate: only five patients responded to 311-325 alone, 331-345 alone or the combination. Evidently, some patients responded to an epitope at around 331-335 in the context of that at around 321-325, i.e. on the same peptide. Peptide 321-335 induced proliferation in 11 of the 24 patients. Other patients (12/24) responded to the same 331-335 epitope in the context of later sequence, via peptide 331-345. The simple combination of peptides 321-335 and 331-345 induced responses in only 8/24. The other 8 of the positively responding patients needed to see the 311-325 peptide. These results explain the finding underlying the invention that a three peptide combination is required if reasonably short peptides are to be used.

A further 41 patients with cervical disease were tested and compared to 11 disease-free controls. The results are shown in Table 2 below.

TABLE 2

Proliferative T-cell responses of patients' short term lines to β-gal HPV 16.L1 fusion protein (aa 199-409) and to synthetic peptides (7), (9) and (11).

Stimulating antigen	Number of subjects expressed as a percentage giving >109% response	
	Patients (n = 41)	Controls (n = 11)
β-gal HPV 16.L1	100	100
311-325 (7)	27	9
321-335 (9)	27	0
331-345 (11)	29	9

All the patients responded to HPV 16.L1 in 10 or more of their cell lines and responses to all the peptides were detected. It was found that the greatest number of responding cell lines per patient was directed against peptide (11) followed by peptide (9).

Cervical biopsy tissue was obtained for clinical diagnostic purposes from the 41 patients at the same colposcopy clinic as the samples of blood used in the assays above were collected. These biopsies were histologically graded independently and without prior knowledge of the above T-cell data. Additional biopsy sections were cut at a later time from selected blocks which had evidence of disease activity to detect and type any HPV DNA present by PCR.

The results are shown in Table 3 below.

TABLE 3

Patient proliferative responses, HPV types and cervical biopsy histology

HPV type	Type of lesion	n	No of Proliferative T-cell responses
X	Atypical	6	2
	CIN I/II	5	2
	CIN III	1	1
HPV 16	Atypical	0	-
	CIN I/II	1	1
	CIN III	11	11
HPV 18	Atypical	1	0
	CIN I/II	1	0
	CIN III	1	0
HPV 33	Atypical	0	-
	CIN I/II	1	1
	CIN III	0	-
HPV-ve	Atypical	3	3
	CIN I/II	6	4
	CIN III	4	1

HPV DNA typing of the 41 biopsies identified 13 (31.7%) cases with no detectable HPV DNA, 12 (29.3%) with an unidentifiable (type X) HPV DNA type (i.e. HPV DNA positive but not HPV types 6, 11, 16, 18, 31 or 33) and a further 12 patients (29.3%) were HPV 16 positive (1 had a mixed infection with HPV 16 and 18). Of the remaining 4 patients, 3 (7.3%) were HPV 18 positive and 1 (2.4%) was HPV 33 positive. The patients biopsies were also grouped into one of three histological categories, namely atypical, CIN I and II or CIN III. In the atypical group (n=10), the most prevalent HPV types identified were type X (60%) while 33% of biopsies were found to be HPV negative. A similar pattern was found with CIN I/II grade biopsies where 42% were HPV negative and 35% had HPV type X. In the high grade CIN III group (n=17), the majority of patients typed had HPV 16 (64%). There is, therefore, a strong association between HPV 16 DNA positive patients and CIN III grade lesions (P=0.0001). However, there were patients (23%) in this group in whom no HPV DNA could be found. No significant correlation was found between those patients who were HPV DNA positive (irrespective of HPV type) and histological grading. In those where HPV 18 was found all three histological grades were represented, and the single patient with HPV 33 had a CIN II grade lesion, but the patient numbers were small (n=4).

Also illustrated in Table 3 are the patients proliferative T-cell responses to HPV 16.L1 peptides, their cervical biopsy histology and HPV DNA typing results. In the HPV DNA negative group of patients there were responders (n=8) found in all three categories of cervical lesions with 7/8 being associated with the atypical and low grade CIN I/II lesions. Responders who were HPV DNA positive were found in three of the four HPV type categories; HPV X (n=5), HPV16 (n=12) and HPV33 (n=1). No proliferative T-cell responses were detected in the HPV 18 positive group. The most striking finding was that all the patients who were HPV 16 positive responded to one or more peptides and 92% of them had CIN III lesions. The remaining responder had a CIN I/II lesion and no patients were found in the atypical group. Statistical analysis by Chi-squared test revealed a highly significant

association between responders and the presence of HPV16 DNA ($p=0.0004$). It has not been possible to show an association between T-cell responses in individuals who were HPV16 DNA positive and their histological grade because of the absence of non-responders. However, there was an association between T-cell responses of individuals who were HPV DNA positive and the histological grade of their lesions ($p=0.004$).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Synthetic Peptides of Human Papillomavirus

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25(EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9313556.1
(B) FILING DATE: 01-JUL-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Asn Leu Ala Ser Ser Asn Tyr Phe Pro Thr Pro Ser Gly Ser Met
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Pro Ser Gly Ser Met Val Thr Ser Asp Ala Gln Ile Phe Asn Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gln Ile Phe Asn Lys Pro Tyr Trp Leu Gln Arg Ala Gln Gly His
1 5 10 15

CLAIMS

1. Peptides for conjoint use in therapy of patients having an infection caused by HPV 16 or a like human papillomavirus, or in which such a HPV has been implicated, said peptides being from 15 to 24 amino acids long and consisting of or including the following sequences of amino acids of human papillomavirus (HPV) 16 L1 protein:

(1) SEQ ID. NO:1 = aa residues 311-325:

Asn	Leu	Ala	Ser	Ser	Asn	Tyr	Phe	Pro	Thr
1				5					10
Pro	Ser	Gly	Ser	Met					
				15					

or a conservatively modified variant thereof;

(2) SEQ ID. NO: 2 = aa residues 321-335:

Pro	Ser	Gly	Ser	Met	Val	Thr	Ser	Asp	Ala
1				5					10
Gln	Ile	Phe	Asn	Lys					
				15					

or a conservatively modified variant thereof; and

(3) SEQ ID. NO: 3 = aa residues 331-345:

Gln	Ile	Phe	Asn	Lys	Pro	Tyr	Trp	Leu	Gln
1				5					10
Arg	Ala	Gln	Gly	His					
				15					

or a conservatively modified variant thereof.

2. Peptides according to Claim 1 wherein any one or more of them is from 15 to 22 amino acids long.

3. Peptides according to Claim 2 wherein any one or more of them is from 15 to 18 amino acids long.

4. Peptides according to Claim 1, 2 or 3 wherein any additional sequence beyond that shown in Claim 1 is contiguous sequence from HPV 16 L1 protein.

5. Peptides according to any preceding claim in the form of conjugates to carrier or assistant molecules.

6. Peptides according to any preceding claim for conjoint use in the therapy of human patients having cervical dysplasias.

7. A therapeutic composition comprising each of the peptides defined in any one of claims 1-5.

8. A composition according to Claim 7 which further includes a therapeutically acceptable carrier or adjuvant.

9. A composition according to Claim 7 or 8 for use in therapy as defined in Claim 1.

10. A composition according to Claim 7 or 8 for use in therapy of human patients having cervical dysplasias.

11. One to three peptides for single or conjoint use in therapy of patients having cervical dysplasias, the or each peptide individually being from 15 to 35 amino acids long and consisting of or including a sequence of amino acids of human papillomavirus (HPV) 16 L1 protein, the peptide, or peptides considered together, providing HPV 16 L1 amino acid sequence consisting of the whole of the sequence from amino acids 311-345 (without contiguous N- or C-terminal sequence of HPV 16 L1 beyond 311 or 345 respectively).

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 94/01397

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/025 A61K38/10 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 02184 (THE UNIVERSITY OF QUEENSLAND) 4 February 1993 see page 19, line 8 - line 22; claims; tables 1,no.32-34 ---	1-11
X	WO,A,90 04790 (MEDSCAND AB) 3 May 1990 see claim 1; tables 3,no.21-23 ---	1-6,11
A	JOURNAL OF GENERAL VIROLOGY vol. 71, no. 2 , February 1990 pages 423 - 431 G. STRANG ET AL 'Human T cell responses to human papillomavirus type 16 L1 and E6 synthetic peptides: identification of T cell determinants, HLA-DR restriction and virus type specificity' see Discussion on pages 429-430 -----	1-11

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 1994	-4 -10- 1994

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. ional Application No
PCT/GB 94/01397

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9302184	04-02-93	AU-B-	651727	28-07-94
		EP-A-	0595935	11-05-94
WO-A-9004790	03-05-90	AU-B-	639666	05-08-93
		AU-A-	4481589	14-05-90
		DE-D-	68912342	24-02-94
		DE-T-	68912342	05-05-94
		EP-A-	0440700	14-08-91
		JP-T-	4506562	12-11-92
		SE-A-	8803870	28-10-88